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FORMATION OF COMPLEMENTARY RNA AND ENZYMES IN *BACILLUS SUBTILIS* INFECTED WITH BACTERIOPHAGE SP8

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The properties of messenger RNA (m-RNA) have been deduced from a variety of systems. Bautz (1963) has presented evidence favoring a single stranded nature of bacteriophage specific m-RNA. Genetic evidence has been obtained by Champe and Benzer (1962) with the same coliphage system suggesting that the template for useful messenger RNA synthesis is one of the two complementary bacteriophage DNA strands. However, *in vitro* experiments show that both strands of partially degraded DNA are copied by the DNA-primed RNA polymerase (Geiduschek *et al.*, 1961; Hurwitz, 1963). The availability of bacteriophages whose DNAs yield strands which can be fractionated and identified by their different buoyant densities has made it possible to test directly whether the RNA formed after phage infection is complementary to one or both of the strands of the bacteriophage DNA. A preliminary note has summarized our work on one of these bacteriophages, SP8, which is virulent for *B. subtilis* (Marmur and Greenspan, 1963). Some of the properties of SP8 DNA and the consequences of infection upon host biosynthesis will be

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presented in Section I of this paper; Section II will deal with DNA-RNA hybridization studies and induction of a hostspecific enzyme and Section III will be concerned with the formation of bacteriophage-specific enzymes.

I. Properties of SP8 and infected *B. subtilis*

A number of bacteriophages contain DNA which provide two strands of differing density when they are denatured. The bacteriophages are presented in Table I. It is evident from Table I that the majority of those bacteriophage DNAs which have a guanine plus cytosine (G + C) content of less than 50% often possess complementary strands of dissimilar buoyant densities whereas other bacteriophages (not listed) with G + C contents of greater than 50% do not display this feature (Marmur and Cordes, 1963).

We have selected for study bacteriophage SP8 (Romig and Brodetsky, 1961) which is virulent for *B. subtilis* Marburg. SP8 produces prompt and reproducible lysis of host suspensions grown in broth and defined media and yields clear plaques on solid media. Its chemically analyzed base composition is similar to that of the host and contains 43% G + C. From electron microscopy studies of bacteriophage SP8 and studies of the sedimentation behavior of its unsheared DNA complement, there appears to be a similarity in size to the T-even *Escherichia coli* bacteriophages (P. F. Davison, personal communication). The banding patterns of bacteriophage SP8 DNA in its native and denatured states and of its complementary strands (fractionated from each other by chromatography on a methylated albumin-kieselguhr (MAK) column) are seen in Fig. 1. The strand with the higher buoyant density in the CsCl gradient (the H or « heavy » strand) has a larger band width than does the complementary light (L) strand, and may have a lower molecular weight. This relationship has been observed in a majority of the banding patterns of denatured and fast cooled SP8 DNA preparations. The same is true of *Clostridium tetani* bacteriophage DNA as well as several other bacteriophage DNAs listed in Table 1. The lower molecular weight may result from pre-existing backbone chain scissions predominantly on the heavy DNA strand, or may have been introduced

TABLE 1

The Buoyant Densities and Base Compositions of Bacteriophage DNAs Yielding Bimodal Bands in CsCl Gradients when Denatured

Bacteriophage	Host	Buoyant Density gm/cc Native	Denatured		G + C from Native DNA T_m	Density
SP6	*B. subtilis*	1.743	1.755,	1.761	17.5	84
SP7	»	1.743	1.755,	1.760	17.5	84
SP8	»	1.743	1.756,	1.762	17.5	84
SP9	»	1.743	1.755,	1.764	17.5	84
SP13	»	1.743	1.755,	1.766	17.5	84
SP5	*B. licheniformis*	1.742	1.754,	1.761	17.5	83
G	*B. megaterium*	1.701	1.710,	1.727	40	41
A	*B. cereus*	1.694	1.710*		37	35
B	»	1.695	1.711*		37	36
C	»	1.696	1.711,	1.719	37	37
D	»	1.696	1.710*		37	37
α	*B. tiberius*	1.705	1.717,	1.726	41	45
SA	*M. pyogenes* var. *aureus* 77	1.693	1.706,	1.714	35	34
CT1	*Clostridium tetani*	1.691	1.703,	1.716	32	31
PR-1001	*Agrobacterium radiobacter*	1.712	1.724,	1.729	—	53

* Although two bands are clearly visible on the ultraviolet absorption photograph, their resolution by microdensitometer tracing is difficult. The denatured buoyant density reflects the average of the light and heavy bands which are estimated to differ by about 0.003 gm/cc.

TABLE 1. - The buoyant densities were determined as described previously (Schildkraut et al., 1961) using *E. coli DNA* (1.710 gm/cc) as a reference standard. The T_m was determined in 0.15 M NaCl plus 0.015 M Na citrate (SSC). *B. subtilis* bacteriophages SP6, SP7, SP8, SP9 and SP13 were obtained from W. R. Romig; SP5 from C. B. Thorne (1962); bacteriophage G from L. Siminovitch (Murphy, 1957); *B. cereus* bacteriophages A, B, C and D from J. R. Norris (1961); phage α is described in Cordes et al., (1961); the *M. pyogenes* var. *aureus* bacteriophage from R. C. Cleverdon, and *Cl. tetani* phage from M. Mandel, W. C. Latham and R. J. Timperi; phage PR-1001 is described in Rozlycky et al., (1963).

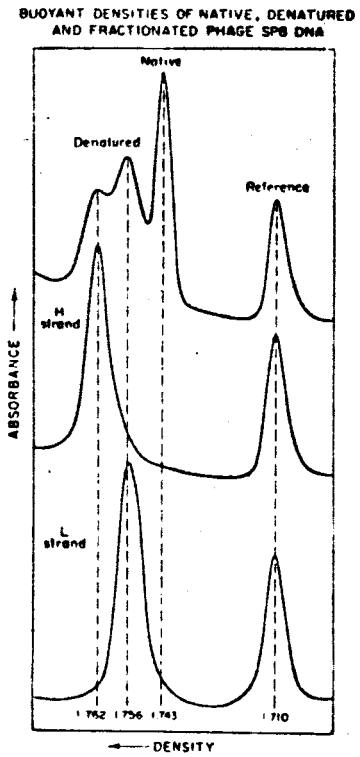

Fig. 1. — Buoyant Densities of Native, Heat Denatured and Fractionated Strands of SP8 DNA.

The buoyant densities were determined as described previously (Schildkraut et al., 1961) using *E. coli* DNA (1.710 gm/cc) as a reference standard. SP8 DNA in this and other experiments was isolated from concentrated bacteriophage lysates in broth using phenol extraction followed by a final deproteinization with chloroform-isoamyl alcohol and precipitation and washing with ethanol. The strands of heat denatured SP8 DNA were fractionated on a single layer MAK column using a selective three-step elution procedure with increasing concentrations of NaCl in 0.05 M phosphate buffer (SP) at pH 6.7 (Sueoka and Cheng, 1962). The L strand eluted at about 0.85 M SP and the H strand at higher ionic strength. The effluent fractions were monitored by absorbance measurements at 260 mμ, and the identity and purity of the strands determined by analytical CsCl density gradient centrifugations as illustrated above. The purified fractions were concentrated by evaporation and dialyzed into the appropriate solvent (2xSSC usually). Samples containing at least 95% of the particular strand were considered pure and used in succeeding experiments.

during the isolation and purification of the bacteriophage DNA. The bacteriophage DNA samples which yield only a single band in the CsCl gradient in the denatured state are listed in Table 2. Not listed in Table 2 are DNA preparations isolated from bacteriophages P22, λ and the *E. coli* T-phages which similarly band within a unimodal peak in the denatured state.

It is apparent that the SP8 L DNA strand differs in base composition from the H strand and they are presumably comple-

TABLE 2

Native Buoyant Densities and Base Compositions of Denatured Bacteriophage DNA Yielding Unimodal Bands in CsCl Density Gradients

Bacteriophage[a]		Host	Native Buoyant Density (gm/cc)	G + C from Buoyant Density
S24V	(1)	*Serratia marcescens*	1.714	55
1X1	(2)	*Pseudomonas aeruginosa*	1.722	63
PRM1	(3)	*Rhizobium meliloti*	1.708	49
MSP8	(4)	*Streptomyces griseus* S104	1.729	70
N1	(5)	*M. lysodeikticus*	1.724	65
PBS1 and 2	(6)	*B. subtilis*	1.722	63[b]
1KT, 6a, 6b, 4P, NT	(7)	»	1.722	63[b]
SP3	(8)	»	1.694	35
SP10	(9)	»	1.714	55[b]
P-XP5	(10)	*Xanthomonas pruni*	1.721	62

[a] The numbers in parenthesis listed to the right of the bacteriophage represents the source of (and in some cases published reference to) the phage. (1) M. Mandel; (2) C. D. Grabar;; (3) I. Takahashi; (4) L. A. Jones; (5) Scaletti and Naylor (1959); (6) Takahashi (1963); (7) Ivanovics and Csiszar (1962); (8) Romig and Brodetsky (1961); (9) Thorne (1962); (10) A. Eisenstark.

[b] The high G plus C contents, when compared to those estimated from the thermal denaturation temperature values, are indicative of the presence of unusual base.

TABLE 2. - The buoyant densities were determined as described previously (Schildkraut et al., 1961) using *E. coli* (1.710 gm/cc) as the reference standard.

mentary in their base composition (Table 3). As in the case of bacteriophage α (Marmur and Cordes, 1963), the H strand is

TABLE 3

Ratio of Bases in DNA Calculated from Ratio of their Complementary Nucleotides Incorporated into DNA-Primed RNA

Radioactive ribonucleotides	$\frac{UMP-P^{32}}{CMP-H^3}$	$\frac{AMP-H^3}{UMP-P^{32}}$	$\frac{GMP-P^{32}}{CMP-H^3}$
Corresponding base pair in DNA	$\frac{A}{G}$	$\frac{T}{A}$	$\frac{C}{G}$
DNA used as primer			
Calf thymus	(1.27)	(1.00)	(1.00)
SP8-NATIVE	1.33	0.86	0.93
SP8-LIGHT STRAND	1.21	0.78	0.87
T2-NATIVE	1.85	1.01	0.97
PBS2-NATIVE	2.27	0.90	0.97

TABLE 3. - Base ratios of small quantities of DNA were estimated by measuring the ratio of incorporation of each of the 4 ribonucleotides into RNA whose synthesis was catalyzed by RNA polymerase (purified from *E. coli* W) in reaction mixtures containing one of these DNAs as primer. Pairs of ribonucleoside triphosphates, distinguishable from each other by their isotopic markers, were employed in each reaction mixture such that the ratio of their incorporation represented: in column I the approximate $\frac{A+T}{G+C}$ ratio, in column II the ratio of the complementary base pair T:A, in column III the ratio of the complementary base pair C:G. To eliminate any need for determining the exact specific radioactivities of the substrates, the results were expressed in relation to those obtained when calf thymus DNA was used as primer (after the incorporation ratios obtained with the latter DNA were expressed in terms of che chemically established base ratios).

Reaction mixtures for the above experiments consisted of 0.5 ml volumes containing 40 millimicromoles of each of the 4 ribonucleoside triphosphates: ATP, GTP, CTP and UTP. Where indicated, non radioactive triphosphates were replaced by ATP-H^3 (containing 900 c.p.m. per mμ mole). In addition, each reaction mixture contained 2 μ moles of $MnCl_2$, 4 μ moles of $MgCl_2$, 25 μ moles of Tris buffer, pH 7.5, 1 μ mole of β mercaptoethanol, an amount of one of the indicated DNAs containing 50 mμ moles of nucleotide phosphorus, and 2 μg of the ammonium sulfate II B fraction of RNA polymerase (Furth et al., 1962). The reaction mixture was incubated at 38°C for 20 minutes, at which time the extent of incorporation (usually a total of 5 mμ moles of ribonucleotides) was measured by precipitating the synthesized RNA with 5% trichloracetic acid and recovering the acid insoluble material on a HA Millipore filter. The filter was immersed in a scintillation

relatively pyrimidine-rich and the L strand is enriched in purines. The inequality of the Watson-Crick base pairs in the fractionated

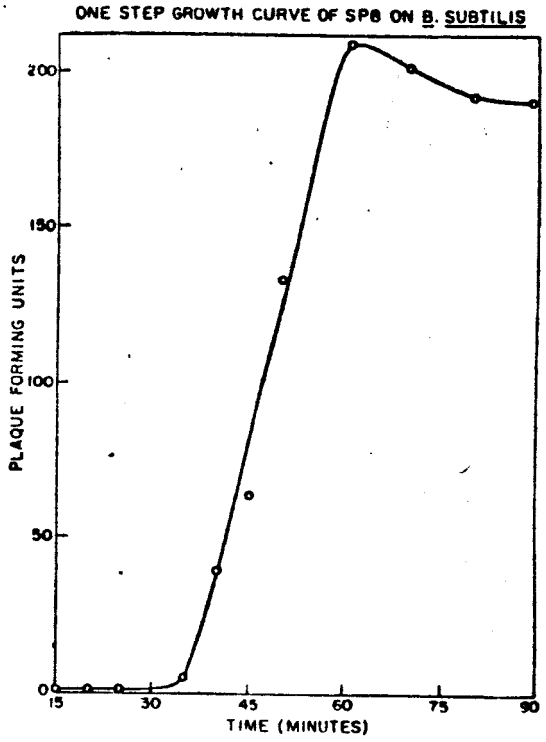

Fig. 2. — One Strep Growth Curve of Bacteriophage SP8 on *B. subtilis* Marburg.

The one step growth curve was performed as described by Adams (1959). Antiserum against SP8 was kindly supplied by Dr. L. Levine. Platings were made on Difco Penassay plates with tryptone-yeast extract (Romig and Brodetsky, 1961) soft agar overlay, using *B. subtilis* spores as plating bacteria (Romig, personal communication). Infective centers, almost 100%, m.o.i. 6.7. In this and subsequent experiments the cells were grown and infected at 37° C in a reciprocating water bath.

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fluid (containing toluene as the only solvent), and its radioactivity was counted in the appropriate channels of a Packard Tri-Carb scintillation spectrometer.

The inequality of incorporation of complementary nucleotides in reactions primed by native DNA is regarded as significant. This asymmetry, which presumably reflects a preference of the RNA polymerase for the light DNA strand of the native helix, has also been observed with the DNA of the *B. subtilis* phage PBS2 (Takahashi, 1963) but, as shown above, is not evident in reactions primed by T2 DNA.

strand tends to discount the possibility that each is a double stranded sub-unit of the native preparation.

Since the consequences of bacteriophage SP8 infection of *B. subtilis* have not previously been described, we have presented (Figs 2-6) some of the characteristics which are most pertinent to this study. Figure 2 shows the one step growth curve obtained in broth medium, the latent period is approximately 35 minutes and the burst size is 200/cell. All cells infected with SP8 lyse and a great majority give rise to a productive burst. None of the survivors of the infection have been found to be lysogenic.

The relative amounts of host and bacteriophage DNAs in infected cells were studied at various times up to bacteriophage-

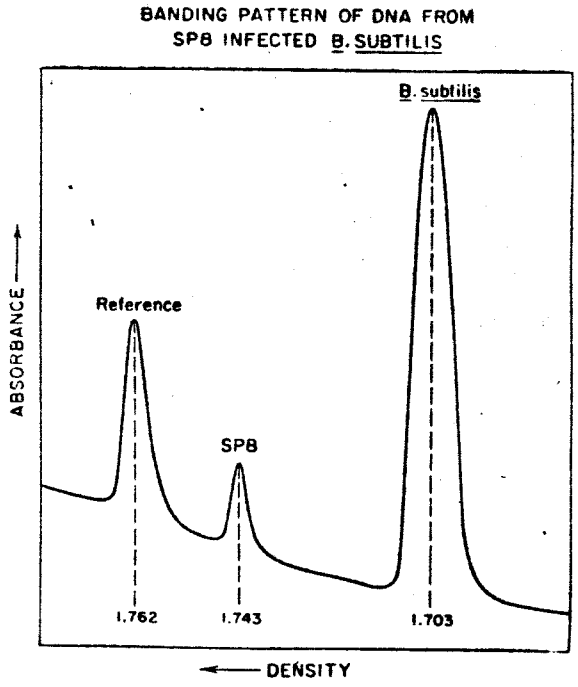

Fig. 3. — Banding Pattern of a Partially Purified DNA Extract of SP8 Infected *B. subtilis*.

Ten minutes after infection the cells were chilled, collected, lysed, deproteinized once with chloroform-isoamyl alcohol (Marmur, 1961) and treated with RNAase. The reference standard is deuterated *Pseudomonas aeruginosa* DNA (1.762 gm/cc). When the reference standard is omitted, no SP8 is found to band with a buoyant density within that of the reference DNA range.

induced lysis by examining the banding patterns of partially purified lysates in CsCl density gradients. Host DNA was also examined for its transforming activity in the same nucleic acid extracts. A typical CsCl density gradient banding pattern of DNA extracted from SP8 infected *B. subtilis* cells is shown in Fig. 3. The characteristically high buoyant density of the bacteriophage DNA (1.743 gm/cc) readily distinguishes it from host cell DNA (1.703 gm/cc). This is most likely due to the replacement of the thymine residues by hydroxymethyl uracil (HMU) and the partial glucosylation of the cytosine residues (Mahler, unpublished work quoted in Takahashi and Marmur, 1963). The tracing in Fig. 3,

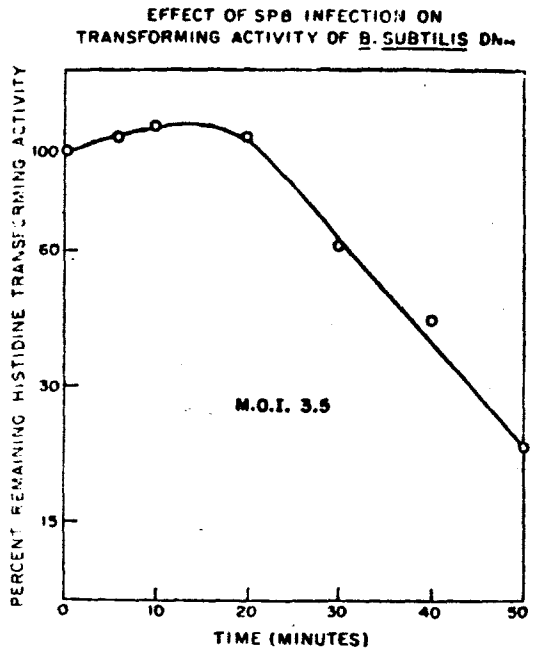

Fig. 4. — Fate of *B. subtilis* DNA in SP8 Infected Cells.
Cells were grown and infected in Difco Penassay broth. DNA was prepared in a partially purified state from cells collected at various time intervals by the procedure of Marmur (1961). The lysate was deproteinized once with chloroform-isoamyl alcohol and the ethyl alcohol steps were omitted. The nucleic acid preparation was treated with RNAase and then dialyzed against 0.15 M NaCl plus 0.015 M Na citrate (SSC). Transformations were performed as previously reported (Marmur et al., 1963; Mahler et al., 1963) using a histidine auxotroph as the recipient cell. Visible lysis of the culture occurred in 60 min.

as well as those of other banding patterns of DNA samples extracted from *B. subtilis* at various times after infection, displays one important feature: the buoyant density of the SP8 DNA in the CsCl density gradient is always the same and similar to that of native DNA extracted from mature bacteriophage particles. At no time did DNA isolated from infected cells yield the two band pattern characteristic of denatured SP8 DNA.

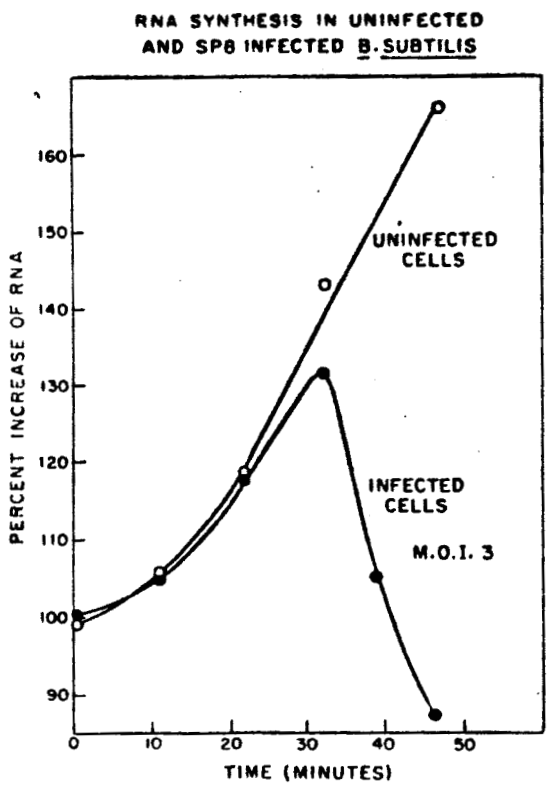

Fig. 5. — Total RNA Synthesis Following SP8 Infection.
Cells were grown and infected with SP8 in a Tris synthetic medium similar to that described by Nomura et al., (1963). The medium consisted of 0.1 M Tris, pH 7.4; 0.08 M NaCl; 0.02 M KCl; 0.02 M NH_4Cl; 6.4×10^{-4} M KH_2PO_4; 1.6×10^{-4} M Na_2SO_4; 10^{-3} M $MgCl_2$; 10^{-4} M $CaCl_2$; 10^{-5} M $FeCl_3$; 0.1% glucose and supplemented with tryptone-yeast extract (Romig and Brodetsky, 1961), 10 ml/liter. Samples of infected and uninfected cell suspensions were first treated with 0.2 M $HClO_4$, centrifuged and the RNA hydrolyzed with 1 M $HClO_4$ (Richmond, 1959). RNA was estimated colorimetrically by the orcinol method (Schneider, 1957). Visible lysis of the culture occurred in 48 min.

The amount of each DNA constituent was estimated from the area within the relevant band of the microdensitometer tracings made from the ultraviolet absorption photographs. Bacteriophage DNA appears 5 minutes after infection and increases in amount in a linear fashion from the 10th minute until the time of the burst. The amount of host cell DNA increases slightly at first and

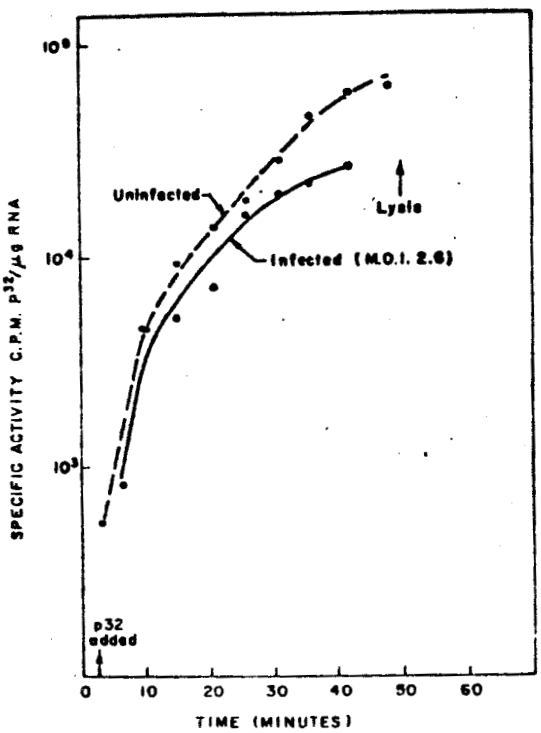

Fig. 6. — Effect of SP8 Infection on P^{32} Incorporation into *B. subtilis* RNA. Cells of *B. subtilis* were grown and infected with bacteriophage SP8 in the Tris synthetic medium listed in the legend to Fig. 5, supplemented instead with neopeptone (20 mg per cent) to give a final concentration of 80 mμ moles/ml of inorganic phosphate. Carrier-free P^{32} (4 mc/350 ml culture) was added to the infected and control cultures 2.5 min after infection. Samples of each culture (20 ml) were removed at the times indicated and added to 5 ml of crushed frozen acetate buffer, pH 5.2. RNA was isolated following lysis by the combined treatment of the washed cells first with lysozyme and then with sodium lauryl sulfate using a modification of the method of Okamoto et al. (1962). The specific activity was determined from the amount of cold TCA precipitable radioactivity relative to the absorbance at 260 mμ. Radioactivity was assayed by scintillation as described in the legend to Table 2.

then declines gradually. In parallel experiments each of the lysates employed in the CsCl density gradient banding experiments was examined for its ability to transform histidine-requiring cells of *B. subtilis* to a prototrophic state. As shown in Fig. 4, there is an initial increase in transforming activity, followed again by a gradual decrease, most likely reflecting a slow disintegration of bacterial DNA close to the time of lysis. The host DNA retains not only transforming activity but functionality as well, since RNA synthesis continues at a normal rate over most of the time course of infection (Fig. 5). The increase in turbidity of the infected culture is unaffected for about 15 minutes after phage infection. Similarly, the rate of P^{32}-labeling of RNA in infected *B. subtilis* parallels that of uninfected cells (Fig. 6).

II. Specificity of Hybridization of DNA with Messenger RNA

The experiments described in Section I indicate continued function by the host genome after infection with SP8. In this respect, the behavior of infected cells resembles that of *E. coli* lytically infected with bacteriophage λ (Siminovitch and Jacob, 1952). It contrasts with the complete cessation of host-specific synthesis in *E. coli* infected with the virulent T-even coliphages. Bacterial messenger RNA is presumably synthesized together with phage specific m-RNA. In order to demonstrate that RNA complementary to two different species of DNA (bacteriophage and host) is synthesized in SP8 infected *B. subtilis*, use was made of the principle of DNA-RNA hybridization described by Spiegelman and Coworkers (1961, 1963) which was used to isolate and identify the specific m-RNA. The use in separate hybridizations of each of the complementary strands of SP8 DNA permitted us to ascertain which of the phage strands served as a template for the synthesis of complementary RNA *in vivo*.

In most of our studies the annealed hybrid DNA-RNA molecules were separated by preparative CsCl density gradient centrifugation (Spiegelman, 1961). Hybrids were formed by annealing labeled RNA isolated from SP8 infected *B. subtilis* with the H and with the L SP8 DNA strands previously separated chromatographically on a MAK column. After preparative centrifugation of the hybridization mixture in a CsCl density gradient,

drops were collected from the bottom of the punctured centrifuge tube and assayed for acid precipitable radioactivity (after pre-incubation either with or without ribonuclease (RNAase)). In Fig. 7 the extent of hybridization of the H and of the L SP8 DNA strands with phage infected *B. subtilis* RNA is compared. Results obtained from samples which were treated with RNAase indicate clearly that only the RNA hybridized with the H SP8 DNA strand has the RNAase resistance expected of DNA-RNA hybrids (Giacomoni and Spiegelman, 1962). The amount of RNAase-resistant, acid-precipitable radioactivity which renatured with the H strand represents 10-15% of the labeled RNA added to the annealing mixture. On the other hand, the radioactivity associated with the L SP8 DNA strand is about 0.5% of that added to the annealing mixture and could reflect contamination with H strand of the L strand preparation used for annealing. The shoulder on the light density side of the H strand-RNA hybrid (Fig. 7) is not seen with a more highly purified H strand preparation. In each case, when a small amount of radioactivity was found at a lower density, it was presumed to be due to RNA hybridized together with imperfectly renatured bacteriophage DNA as a consequence of the minor contamination with the complementary strand.

Experiments in which the total radioactivity of the annealed DNA-RNA hybrids collected from the CsCl gradient was assayed without prior RNAase treatment (Fig. 8) gave approximately similar results to those shown in Fig. 7, as did a similar experiment not employing other RNAase or acid-precipitation. A distinct peak of radioactivity was evident only when the H strand was used and supports the above conclusions.

When hybridizations were performed with denatured, but unfractionated SP8 DNA, less RNAase resistant radioactivity was annealed to the DNA than could be recovered with fractionated SP8 DNA strands. This is probably the result of a preferential renaturation of the complementary SP8 DNA strands with each other.

To eliminate the objection that the L SP8 DNA strand is intrinsically unable to hybridize with complementary RNA, each

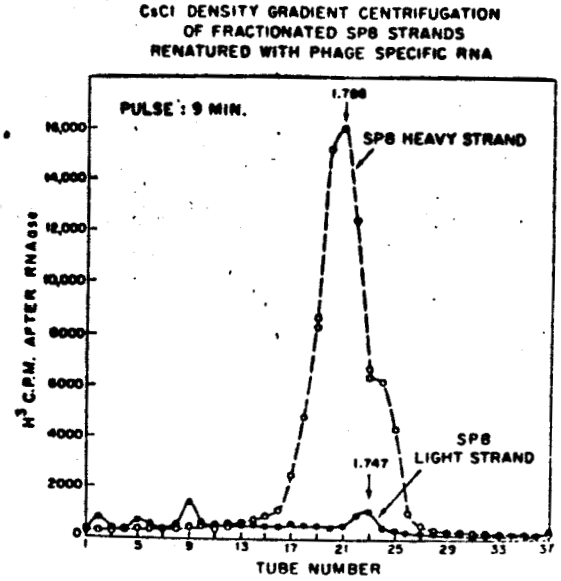

Fig. 7. — Specificity of Hybridization of RNA Isolated from SP8 Infected *B. subtilis* with the Fractionated Strands of SP8 DNA.

The hybridization mixtures, in 2 × SSC, consisted of 32 μg H³-uridine labeled RNA (6×10^6 c.p.m./μg cold TCA precipitable) and 10 μg each of the fractionated L and H SP8 DNA strands. Total volume was 0.55 ml. The mixtures were annealed at 57° C for 10 hrs followed by slow cooling to room temperature. Solid optical grade CsCl (Maywood) and water were added to yield a density of 1.74 gm/cc in a final volume of 3 ml. The samples were centrifuged at 33,000 r.p.m. for 3 days at 25° C in the SW 39 rotor of a Spinco Model L ultracentrifuge. Fractions were obtained by piercing the lower end of the tube and collecting samples of 3 drops. After every 5-8 drops, the refractive index of one drop was measured. To each 3-drop sample was added 2 ml of water containing 10 μg RNAase and 50 μg calf thymus DNA (Worthington) as carrier. Following 22 min incubation at room temperature, the nucleic acids were precipitated with cold 20% TCA and collected on HA Millipore filters and the radioactivity assayed by scintillation spectrometry (Hall and Spiegelman, 1961). Densities of the peaks were calculated from the refractive indices.

H³-uridine labeled RNA was obtained from *B. subtilis* infected in the logarithmic phase (m.o.i. 5) in the medium described in the legend to Fig. 5. After pulse labeling (3.5 to 12.5 min after infection), the bulk RNA was isolated using warm phenol by a modification of the method of Okamoto et al., (1962).

The L and H strands of heat denatured SP8 DNA were fractionated by the method described in the legend to Fig. 1.

of the fractionated SP8 strands was annealed with RNA synthesized by highly purified *E. coli* RNA polymerase primed by both the L and the H SP8 DNA strands. The results (not shown) indicate that the RNAs renature primarily with the purified DNA strand which was used to prime their synthesis.

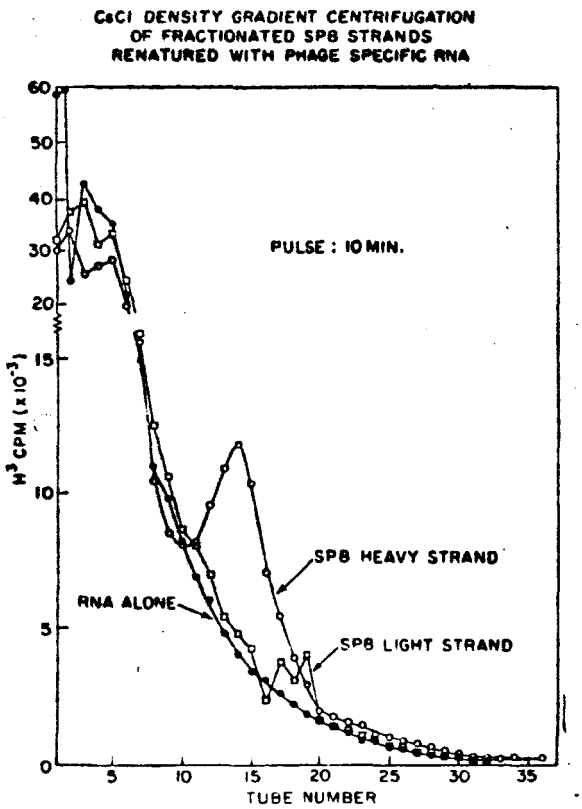

Fig. 8. — Specificity of Hybridization of RNA Isolated from SP8 Infected *B. subtilis* with the Separated Strands of SP8 DNA. Total Acid-Precipitable Radioactivity.

Hybridizations and centrifugations were performed by the procedures described in the legend to Fig. 7. The H^3-uridine-labeled RNA contained 900 c.p.m./μg. The labeling time was from 4 to 14 min following infection. The L strand preparation was further purified by a second passage through a MAK column. Total volume of hybridization mixtures was 0.76 ml. The samples dripped from the centrifuge tubes were diluted to 2 ml with water and calf thymus carrier, precipitated and counted. The scale of the abscissa is different than that of Fig. 7.

The DNA-agar gel technique (Bolton and McCarthy, 1962; McCarthy and Bolton, 1963) was also used to determine the extent of hybridization of the fractionated SP8 DNA strands with pulse labeled RNA. By this procedure, the presence of host specific m-RNA in the RNA isolated from phage infected *B. subtilis* was also assessed. High molecular weight denatured DNA is trapped in the agar gel and renaturation of DNA is minimized since during the hybridization, efficient and preferential renaturation can occur between complementary RNA and the fixed DNA. After the hybridization was carried out, the non-specifically absorbed RNA was washed through in the « front peak ». The second or « back peak » represents the RNA which had annealed to the DNA embedded in the agar. The data presented in Table 4 shows appreciable radioactivity in the acid-precipitable RNA of the back peak when the H strand or denatured SP8 DNA was used but not when the L strand was used in the column. Since the unfrac-

TABLE 4

Total Radioactivity in DNA-RNA hybrids Formed in DNA Agar Gels with Pulse-labeled RNA from B. subtilis *Infected with Bacteriophage SP8*

Denatured DNA	µg DNA in 250 mg agar gel	c. p. m. eluted from « back-peak »
SP8	12	2568
H strand	4.1	1393
L strand	6.3	568
B. subtilis	63	3074
Calf thymus	60	453
None	—	590

TABLE 4. - DNA-agar gels were prepared as described by Bolton and McCarthy (1962) and McCarthy and Bolton (1963). H^3-uridine labeled RNA was the same as that described in Fig. 7. Hybrid RNA-DNA was formed by annealing 250 mg of each agar gel containing the indicated amounts of denatured DNA with 4 µg of labeled RNA at 65° C for 2 hrs in 1 M NaCl. The renatured mixture was washed with 10 portions (10 ml each) of 2 × SSC at 62°C and the « back-peak » then eluted at 72° with seven 10 ml aliquots of SSC/100. The residual column material was dissolved in 5 M $NaClO_4$ at 72° C and showed very little retention of radioactivity. The radioactivity in the cold TCA precipitable material was counted by scintillation as described in the legend to Table 2.

tionated SP8 DNA and the fractionated strands showed different molecular weights, and therefore different degrees of retention by the agar columns, quantitative comparisons of the extent of hybridization are not possible at this time. The results in Table 4 do however show that m-RNA complementary to host DNA is formed in SP8 infected *B. subtilis*.

It was important to establish whether the production of RNA complementary to *B. subtilis* DNA reflects the presence of any extensive nucleotide sequences in SP8 DNA which are congruent to host DNA. DNA-agar gels containing denatured SP8 or *B. subtilis* DNAs were used to test the extent of hybridization of these two species with various denatured, labeled DNAs. As can be seen from Table 5, there is no evidence of any hybrid formation between the host and SP8 DNA under conditions where the homologous pairs yield appreciable hybridization.

TABLE 5

Formation of DNA-DNA Hybrids by B. subtilis *and SP8 DNA*

Unlabeled denatured DNA in agar gel	Sonicated, denatured DNA	Total c. p. m. eluted from « back peak »
B. subtilis	P^{32}-*B. subtilis*	16,600
SP8	»	690
Calf thymus	»	742
No DNA	»	720
B. subtilis	P^{32}-SP8	1,057
SP8	»	11,331
No DNA	»	2,370

TABLE 5. - DNA-agar gels containing 63 (± 4) μg DNA/250 mg gel were prepared as described by Bolton and McCarthy (1962) and McCarthy and Bolton (1963). Labeled *B. subtilis* DNA in SSC/10 was prepared from cells grown in P^{32}-containing dephosphorylated Difco Penassay broth (Countryman and Volkin, 1959). Labeled SP8 DNA was isolated from purified bacteriophage lysates obtained from cells grown in the medium described in the legend to Fig. 5. Each renaturation mixture (4 ml) contained 250 mg of the indicated agar gel containing the entrapped, denatured high molecular weight DNA to which was added labeled, sonicated (30 min in a 10 Kc Raytheon sonic oscillator) and denatured (100° C for 10 min and quickly cooled) DNA; 10 μg *B. subtilis* DNA (10^4 c.p.m./μg) or 2 μg SP8 DNA (2.5×10^4 c.p.m./μg). The annealing conditions, elution from the agar gel column and assay of the label associated with the hybrid were those described in the legend to Table 3.

The demonstration that messenger-type RNA as well as polyribosomes (or polysomes) can be associated with bacterial membranes (Suit, 1963; Schlessinger, 1963) prompted a search for membrane-associated RNA homologous to host and SP8 DNA formed after bacteriophage infection. Table 6 shows the extent of mixed nucleic

TABLE 6

Hybrid Formation between Denatured DNA from B. subtilis *and SP8 and Pulse Labeled RNA Isolated from Membrane-Associated Polysomes of SP8 Infected and Uninfected Cells of* B. subtilis

Denatured DNA		C. p. m. H^3 — RNA Retained in Hybrid	
		SP8 Infected	Uninfected
B. subtilis	10 μg	2,750	6,470
SP8, unfractionated	10 μg	5,250	—
SP8 H	5 μg	9,130	—
SP8 L	5 μg	1,420	—
None		2,015	1,240

TABLE 6. - The indicated amounts of DNA were annealed for 70 min at 62° in 0.5 M KCl plus 0.01 M Tris buffer, pH 7.3 with 10 μg of each RNA sample (total volume, 4 ml). The annealed mixtures were digested with 0.2 μg/ml RNAase at 37° for 20 min, and the DNA-RNA hybrid collected on nitrocellulose membranes as described by Nygaard and Hall (1963). Radioactivity of the dried membranes was measured by scintillation spectrometry.

The labeled RNA was prepared from cells growing in the medium described in the legend to Fig. 9. *B. subtilis* Marburg was infected with bacteriophage SP8 (m.o.i. 2.5) and pulse labeled (4.5 to 13.5 min after infection) with 14.3 μC/ml H^3-uridine. A non-infected aliquot was treated in a similar fashion. The cultures were chilled in an ethanol-dry ice bath and NaN_3 added at a concentration of 2×10^{-4} M. The cells were collected by centrifugation and resuspended in 0.5 M sucrose plus 0.01 M Tris buffer, pH 7.2 containing NaN_3 and incubated with lysozyme for 6 min at 30°. The protoplasts were centrifuged and resuspended in a magnesium containing buffer designed to stabilize polyribosomes (Schlessinger, 1963). Disruption was aided by several cycles of freezing and thawing. The labeled polyribosomes were isolated as described by Schlessinger (1963) and the RNA extracted with warm phenol. The specific activity of the RNA isolated from the polyribosomes of the uninfected cells was 6.6×10^4 c.p.m./μg and that isolated from SP8 infected cells was 5.6×10^4 c.p.m./μg.

acid hybrid formation between DNA from SP8 and *B. subtilis* and RNA isolated from membrane-associated polyribosomes (Schlessinger, 1963) of infected and uninfected cells. The results from this experiment, using the Nygaard and Hall (1963) filtration technique for the detection of DNA-RNA complexes, indicate qualitatively that: less bacterial messenger RNA is synthesized after SP8 infection than in uninfected cells and that the membrane-associated RNA in infected cells again only hybridizes with the H strand of SP8 DNA.

The functionality of the host-specific RNA synthesized after SP8 infection can be ascertained by studying the formation of an inducible enzyme. Two such enzymes were studied, histidase (Hartwell and Magasanik, 1963) and mannitol 1-phosphate dehydrogenase (Liss et al., 1962), yielding similar results. The inducibility of mannitol 1-phosphate dehydrogenase by mannitol in infected and uninfected cells of *B. subtilis* is shown in Fig. 9. The reduced rate of synthesis of this enzyme in infected cells is to be expected from the previous observation (Table 6) that less host-specific, membrane-associated m-RNA is formed in SP8 infected than non-infected cells.

III. Bacteriophage-Specific Enzymes Induced by SP8.

The bacteriophage-specific RNA which appears as early as 10 minutes after infection presumably serves as messenger not only for the synthesis of structural proteins entering the progeny bacteriophage, but also in the formation of the enzymes of nucleotide metabolism which must arise *de novo* to account for the presence of unusual bases in the bacteriophage DNA. The enzymes would be comparable to those which are induced in *E. coli* upon infection with T-even coliphages (Bessman, 1963). In order to elucidate the pathway by which 5-hydroxymethyluracil nucleotides are synthesized, and the mechanism which excludes the natural nucletotide (TMP) from newly synthesized DNA, we have examined the enzymatic activities of xtracts of *B. subtilis* harvested at various times after infection with SP8.

High activities of two enzymes, virtually absent from uninfected cells, hav so far been detected. One of these, thymidine triphosphatase (TTPase) catalyzes the cleavage of TTP to TMP and inorganic pyrophosphate at rates as high as 18 μM/h/mg

protein in the crude extracts of bacteria infected for 20 minutes. Uninfected cells have less than 3% of this activity. In its high activity, and in its function, TTPase resembles the dCTPase

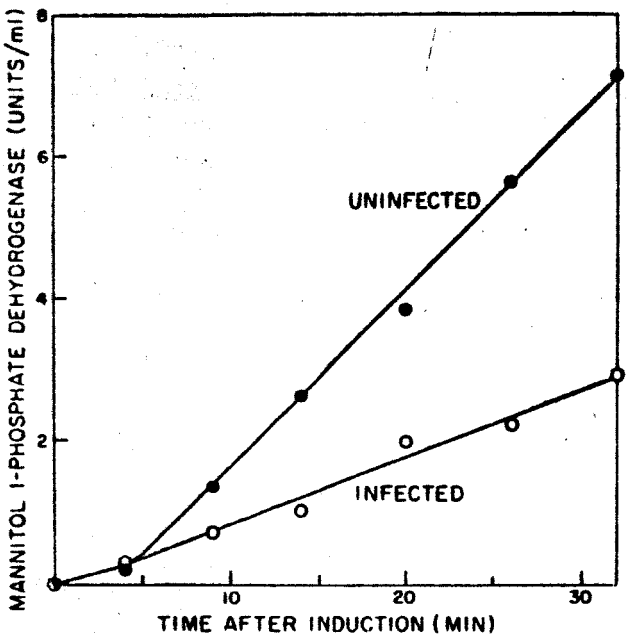

Fig. 9. — Induction of Mannitol 1-Phosphate Deydrogenase in SP8 Infected and Uninfected Cells of *B. subtilis*.

B. subtilis Marburg was grown in the medium described in Fig. 5. It was supplemented with a vitamin-amino acid mixture (Novick and Maas, 1961) instead of tryptone-yeast extract and 0.5% Na glutamate was used as the carbon source instead of glucose. During early logarithmic growth, the culture was divided into two parts and one infected with bacteriophage SP8 (m.o.i. 3). D-mannitol was added (final concentration 0.2%) to both cultures at the same time of SP8 addition. Samples of 7 ml were withdrawn at the indicated intervals, immediately chilled in ethanol-dry ice and harvested by centrifugation. The pellets were resuspended in 2.0 ml 0.05 M Tris-HCl buffer, pH 7.2, containing 20 μg/ml lysozyme. Lysis was carried out for 20 min at 30°, the nucleic acids precipitated with 1% streptomycin sulfate and the supernatant of the centrifuged mixture was assayed for mannitol 1-phosphate dehydrogenase (Liss et al., 1962). The increase in absorbance at 340 mμ (Zeiss spectrophotometer, room temperature) during the 60-120 second interval of the assay was used to measure the reaction rate. One enzyme unit is that amount which will produce a change of 0.01 in absorbance at 340 mμ during the 60-120 second interval.

induced by T-even coliphage infection (Kornberg et al., 1959), which excludes dCMP from bacteriophage DNA containing 5-hydroxymethyl deoxycytidylic acid. It is also comparable, in function, to the dUTPase (Greenberg and Somerville, 1962), present in uninfected *E. coli*, which excludes dUMP from normal DNA. However, unlike these latter enzymes, which cleave their respective nucleoside diphosphates to monophosphates, the SP8-induced TTPase fails to act on TDP.

A second enzyme, dCMP deaminase, converts dCMP to dUMP at rates as high as 25 μM/h/mg protein in crude extracts of infected cells, while those of uninfected cells have no more than 1% of this activity (*). This enzyme does not deaminate 5-methyl dCMP, 5-hydroxymethyl dCMP, dCDP, or r-CMP. It is therefore more specific than the amino hydrolyases of animal tissues (Scarano et al., 1962) which attack 5-substituted deoxycytidylates as well as dCMP. Such high levels of phage-induced dCMP deaminase suggest that the major pathway of ribonucleotide reduction to deoxyribonucleotides in phage infected cells proceeds through dCMP or its phosphates.

While the mechanism excluding TTP from SP8 DNA undoubtedly involves the induced TTPase, the pathway of dHMUMP synthesis remains obscure. It seems unlikely that this nucleotide is derived from dHMCMP since the latter compound is not deaminated by crude extracts of infected *B. subtilis*. (*Note added in proof*. F. Kahan, E. Kahan and B. Riddle have recently described (*Federation Proc., 23*: 318 (1964)) two additional phage-induced enzymatic activities concerned with nucleotide metabolism: (1) *DeoxyUMP hydroxymethylase* adds radioactive formaldehyde to dUMP in the presence of tetrahydrofolic acid a rate of 100 mμ moles/h/mg protein The product after treatment with alkaline phosphatase has the chromatgraphic mobility of hydroxymethyl deoxyuridine. (2) *Deoxy UTPase* hydrolyzes dUTP to dUMP and pyrophosphate at a rate of 25 μ moles/h/mg protein, 10 times the rate of hydrolysis in uninfected cells. They suggest that the dCMP deaminase supplies the dUMP required by the hydroxyme-

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(*) We are grateful to Prof. E. Scarano for suggesting that we search for deaminase activity in phage infected *B. subtilis*.

thylase, while the two deoxynucleoside triphosphatases exclude dUMP an dTMP from viral DNA).

If these enzymes are essential for the formation of SP8 DNA, one would expect them to appear shortly after infection before the bulk of new DNA had been synthesized. This expectation has been confirmed for dCMP deaminase (Fig. 10), for most of the

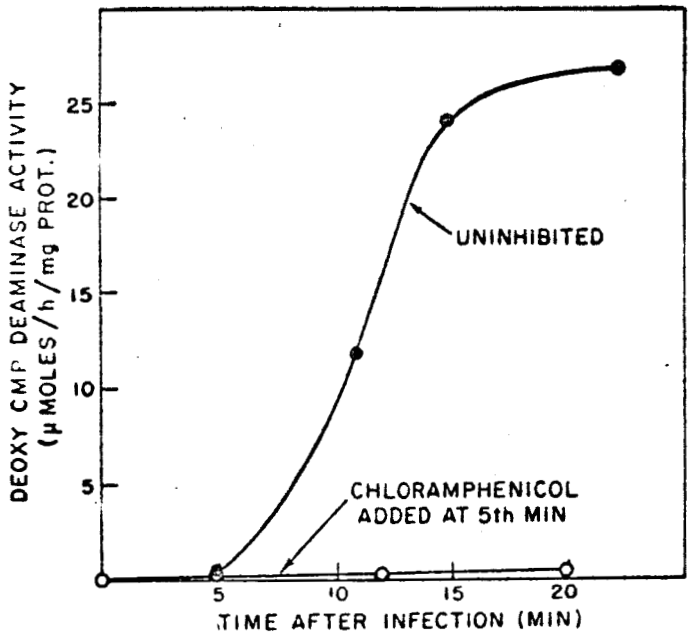

Fig. 10. — Time Course of Formation of dCMP Deaminase in *B subtilis* Marburg Infected with Bacteriophage SP8.

Bacteria were grown in Difco Penassay broth and infected (time zero on figure) at a m.o.i. of 7. Five minutes after infection, 100 μg/ml of chloramphenicol was added to a portion of the infected cells. At the times indicated, 500 ml samples were removed, chilled and centrifuged. The bacterial pellet was ground with alumina and provided 2 ml of crude extract containing 7 mg/ml of protein. DeoxyCMP deaminase was assayed with dCMP-2-C^{14} using the procedure of Maley and Maley (1960).

induced activity appears between the 10th and 15th minute of infection. When chloramphenicol was added to cells 5 minutes after infection, no enzyme activty could be detected at subsequent times. By these criteria, the phage-specific enzymes appear to arise from a *de novo* synthesis of protein, presumably instructed by the bac-

teriophage genome. That these enzymes do not result from an activation, on infection, of the host genome, is indicated by the absence of these activities from extracts of cells infected with another *B. subtilis* bacteriophage, PBS2 (Takahashi, 1963). In this latter case, high levels of two other enzymes not represented in the host are found (Kahan, 1963): a dUMP kinase and a TMP phosphatase. These activities are however related to the composition of PBS2 DNA, for in this case, uracil completely replaces thymine.

Discussion

We have concluded from this study that only one of the two complementary strands of bacteriophage SP8 DNA acts as a template for the synthesis of phage specifc RNA *in vivo*. This conclusion is based, in part, on the ability of approximately 10% of the radioactivity of pulse labeled RNA to form, solely with the heavy strand of SP8 DNA, a hybrid which is resistant to ribonuclease. The same complementarity between RNA and DNA which is held responsible for this resistance to hydrolysis, probably accounts for the retention of radioactive RNA by agar gel columns in which fractionated heavy strand is held immobilized. Conditions of ionic strength and temperature known to denature native DNA are required before the RNA is released. It remains to be shown that the base ratios of the RNA and of the DNA present in the hybrid are complementary to each other.

It is particularly important to establish whether this phage-specific RNA is the same as the messenger RNA which functions as a template for the synthesis of proteins necessary in phage development. It is, for example, conceivable that the truly functional m-RNA (which may be synthesized off the light SP8 DNA strand) turns over so rapidly as it participates in protein synthesis, that its steady state level remains too low to be detected. The product of the heavy strand might accumulate in cells only because it is biologically inert. It was therefore of interest to show that the pulse labeled, membrane-associated RNA, which might be part of the polyribosomes that are the active sites of

protein synthesis (Schlessinger, 1963), hybridizes exclusively with the heavy strand of SP8 DNA.

If in fact the heavy side of the bacteriophage DNA is the only one transcribed into RNA *in vivo*, we must explain how the DNA initially injected into the cell is so polarized or anchored as to prevent the bilateral copying seen in experiments employing purified enzymes. It may be possible to determine structural features of DNA which result in this selective transcription by modifying purified SP8 DNA, either by fractionation or disruption, and then introducing it into the host cell. For these experiments, *B. subtilis* offers the great advantage of being transformable and capable of incorporating into the cell heterologous DNA. Any insight into the mechanism by which only a fraction of a genome is expressed, can ultimately aid our understanding of the factors which confer sequential order upon processes of morphogenesis.

Summary

The DNA of bacteriophage SP8 (whose host is *Bacillus subtilis*) when denatured yields two components of differing buoyant density in cesium chloride density gradients. These components can be separated from each other by chromatography on a methylated serum albumin column. The denser of the two strands, the H strand, contains more pyrimidines and less purines than the lighter L strand. Only the H strand forms hybrids with the pulse labeled RNA synthesized by the infected host. Hybridization has been judged by the ribonuclease insensitivity of the radioactive RNA contained in complexes isolated by preparative CsCl ultracentrifugation, and also by the retention of this radioactive RNA by agar gel columns containing fractionated H or total denatured phage DNA. The L strand has been shown to be capable of annealing with complementary RNA synthesized *in vitro* with it as primer in reactions catalyzed by RNA polymerase.

During the vegetative development of phage, host cells synthesize RNA at normal rates. A small fraction of this RNA forms hybrids with denatured bacterial DNA. Since bacteriophage DNA

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Discussion following Dr. MARMUR's paper.

Dr. Dulbecco: Is the strand separation better or worse at pH 12.5?

Dr. Marmur: We have not investigated in detail the alkali denaturation of our phage DNA preparations. However, at the present time, I believe that the high pH denaturation of DNA (and subsequent reneutralization) is the most gentle method of preparing high molecular weight, single stranded DNA which has suffered little or no degradation in the process. I would like to mention at this point that several other laboratories have been able to demonstrate that the transcription of phage DNA *in vivo* by RNA polymerase involves essentially one DNA strand. This work has been carried out by Spiegelman and his coworkers (*Proc- Natl. Acad. Sci., U. S.*, 50 (1963) 664) who have worked with the *E. coli* phage ØX 174 in its replicative form, as well as by the group (Tocchini-Valentini, Stodolsky, Aurisicchio, Graziosi, Sarnat and Geiduschek, Cold Spring Harbor Symp. Quant. Biol., 1963, in press) who have worked with *Bacillus megaterium* strain Paris phage alpha.

Dr. Reichard: I should like to make a speculative comment on the enzymes which Dr. Marmur talked about. There should be some reason why the dCMP deaminase appears after phage infection and the most straightforward reason I can think of would be to increase the formation of the dUMP and thereby increase the possibility of getting uridine into DNA. There would be so much UMP that the normally occuring dephosphorylation of dUTP would be inadequate so that the dUMP can be pushed into DNA by this enzyme. Consequently, one may suggest that the hydroxymethylation occurs in the DNA after the incorporation of the uridine. In line with this would be that, after all, there are other phage DNA which do contain uridine in the DNA.

Dr. Marmur: The reason that Dr. Kahan has speculated that the formation of the hydroxymethyl uracil was at the diphosphate level of thymidine is that there is no enzyme that phosphorylates the monophosphate of hydroxmethyluridine. Your suggestion that you might get hydroxymethylation at the DNA level has not been checked by us. In some preliminary experiments, however, Dr. Kahan has found that labelled thymidine added to a thymidine requiring strain of *B. subtilis* infected with SP8 is incorporated into the phage DNA. This would tend to argue against the hydroxmethylation of uracil at the level of DNA.

Dr. Cantoni: In connection with HMU and also with the difference in the buoyant density of the SP8 DNA and the *B.* subtilis DNA which you said have the same base composition: is the DNA glucosylation similar to that of T-even phages ?

Dr. Marmur: The glucosylation of SP8 DNA is on the G residues. In PBS2 DNA, the G and C residues are glucosylated.

Dr. Cantoni: And this is the reason for the difference in the buoyant density?

Dr. Marmur: I do not know. It could be preferential binding of cesium.

Dr. Bendich: Do you have any idea where the glucose might be on guanine? Do you think it would be on N_1?

Dr. Marmur: We do not have any chemical data related to your question. It might be in a position that interferes with base pairing since the melting temperature is abnormally low. Instead of being Tm of about 87°C it is down around 70°C.

Dr. Dulbecco: In view of the fact that *Bacillus subtilis* accepts transforming DNA, have you tried to inject the *Bacillus* with either double or single stranded DNA, or the separated phage DNA strands?

Dr. Marmur: We tried to do this experiment. We planned to add to transformable *B. subtilis* cells SP8 DNA in its native, degraded and denatured states (as well as the separated DNA strands) with the view of modifying the DNA *before* cell entry in order to discover if there were any structural features of the DNA which determined the nature of its transcription. However, several preliminary experiments with native DNA convinced us that this experiment was difficult to carry out. In contrast to transformable cells of *Diplococcus pneumoniae*, *B. subtilis* in its transformable state is biochemically « sluggish ». This latent activity makes it difficult to incorporate significant radioactivity into messenger RNA in pulse labeling experiments. Extensive experiments by Stocker and Nester (*J. Bacteriol.* 86 (1963) 785) confirm this biological latency in trasformable *B. subtilis* cultures.